Molecular Biology of the Cell
Vol. 18, 839–849, March 2007

Two Mammalian Sec16 Homologues Have Nonredundant Functions in Endoplasmic Reticulum (ER) Export and Transitional ER Organization[D]

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Submitted August 15, 2006; Revised November 22, 2006; Accepted December 18, 2006
Monitoring Editor: Francis Barr

Budding yeast Sec16 is a large peripheral endoplasmic reticulum (ER) membrane protein that functions in generating COPII transport vesicles and in clustering COPII components at transitional ER (tER) sites. Sec16 interacts with multiple COPII components. Although the COPII assembly pathway is evolutionarily conserved, Sec16 homologues have not been described in higher eukaryotes. Here, we show that mammalian cells contain two distinct Sec16 homologues: a large protein that we term Sec16L and a smaller protein that we term Sec16S. These proteins localize to tER sites, and an N-terminal region of each protein is necessary and sufficient for tER localization. The Sec16L and Sec16S genes are both expressed in every tissue examined, and both proteins are required in HeLa cells for ER export and for normal tER organization. Sec16L resembles yeast Sec16 in having a C-terminal conserved domain that interacts with the COPII coat protein Sec23, but Sec16S lacks such a C-terminal conserved domain. Immunoprecipitation data indicate that Sec16L and Sec16S are each present at multiple copies in a heteromeric complex. We infer that mammalian cells have preserved and extended the function of Sec16.

INTRODUCTION

The transport of newly synthesized secretory proteins from the endoplasmic reticulum (ER) to the Golgi is mediated by COPII-coated transport vesicles (Tang *et al*., 2005; Watson and Stephens, 2005). Vesicle budding is initiated when the transmembrane protein Sec12 recruits the small GTPase Sar1 to the ER membrane. Sar1-GTP recruits the Sec23/Sec24 coat protein complex. The Sec13/Sec31 coat protein complex then binds and apparently polymerizes to create the coat lattice (Stagg *et al*., 2006). Scission of the vesicle is thought to be driven by membrane insertion of an amphipathic N-terminal helix of Sar1 (Bielli *et al*., 2005; Lee *et al*., 2005). Depolymerization of the coat is catalyzed by Sec23, which serves as a GTPase-activating protein for Sar1. These vesicle formation reactions occur at transitional ER (tER) sites, which are ribosome-free ER subdomains (Palade, 1975; Bannykh and Balch, 1997; Mogelsvang *et al*., 2003). tER sites are long-lived but dynamic structures that form de novo and fuse with one another (Bevis *et al*., 2002; Stephens, 2003). However, little is known about how tER sites are created and maintained, or how COPII vesicle budding is restricted to tER sites.

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06–08–0707) on December 27, 2006.

[D] The online version of this article contains supplemental material at *MBC Online* (http://www.molbiolcell.org).

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Abbreviations used: BFA, brefeldin A; GalNAc-T2, *N*-acetylgalactosaminyltransferase-2; GST, glutathione *S*-transferase; tER, transitional endoplasmic reticulum.

As a model system for studying tER sites, we have used the budding yeast *Pichia pastoris*. Unlike the closely related *Saccharomyces cerevisiae*, *P. pastoris* contains discrete tER sites and stacked Golgi organelles similar to those seen in most other eukaryotes (Gould *et al*., 1992; Rossanese *et al*., 1999; Mogelsvang *et al*., 2003). A genetic screen for *P. pastoris* mutants with disrupted tER organization uncovered a role for the 281-kDa Sec16 protein (Connerly *et al*., 2005). In *S. cerevisiae*, Sec16 is a 240-kDa peripheral membrane protein that is essential for ER-to-Golgi transport and cell viability (Espenshade *et al*., 1995). Sec16 interacts with multiple components of the COPII machinery, including the coat proteins Sec23, Sec24, and Sec31 as well as the GTPase Sar1 (Espenshade *et al*., 1995; Gimeno *et al*., 1995, 1996; Shaywitz *et al*., 1997; Supek *et al*., 2002). These various interactions have been mapped to distinct regions of *S. cerevisiae* Sec16. COPII coat proteins colocalize with Sec16 in both *P. pastoris* and *S. cerevisiae* (Huh *et al*., 2003; Connerly *et al*., 2005). It has been suggested that Sec16 serves to nucleate and/or regulate COPII vesicle formation (Shaywitz *et al*., 1997; Supek *et al*., 2002), although the precise role of Sec16 remains to be defined. Sequence comparisons of the Sec16 proteins in *P. pastoris* and *S. cerevisiae* revealed the presence of a central conserved domain and a C-terminal conserved domain (Connerly *et al*., 2005).

We wondered whether mammalian cells contain a Sec16 homologue. Sequence-based homology searches with the central conserved domain of yeast Sec16 identified two related but distinct mammalian genes, both of which are expressed in all tissues examined. One of these genes encodes a large 231-kDa protein that we have designated Sec16L, and the other encodes a smaller 117-kDa protein that we have designated Sec16S. These two Sec16 homologues localize to tER sites. As judged by RNA interference (RNAi)-mediated knockdowns, both proteins are required for ER export and

normal tER organization. In yeast Sec16, the C-terminal conserved domain interacts with Sec23, and a similar Sec23-interacting C-terminal domain seems to be present in Sec16L but not in Sec16S. These results suggest that Sec16L is analogous to yeast Sec16 and that higher eukaryotes have evolved Sec16S as an additional component.

MATERIALS AND METHODS

Nucleic Acid Reagents

Human normal liver total RNA was obtained from Ambion (Austin, TX) (catalog no. 7960). For the experiment of Figure 2C, a panel of cDNAs from major tissue types was obtained from BD Biosciences (San Jose, CA) (catalog no. PT3158-1).

"Stealth" RNAi molecules specific to Sec16L, Sec16S, and Sec12 were from Invitrogen (Carlsbad, CA) and were designed using the company's online program. The RNAi sequences for the experiments shown were GGUUCUGGUGCUUCCGAAAUGGUUU for Sec16L, CCGUGAAGACAGACCAUCUGGUCUU for Sec16S, and CCACUGCAGAAAGUUGUGUGCUUCA for Sec12. Other experiments were conducted with additional RNAi duplexes against Sec16L (GGAUUUGCUAAUAGCCCUGCUGGAA) and Sec16S (UAGUGAAUUUCUCCACGAUCUGCGC). Control experiments were performed with a Stealth RNAi Negative Control Duplex from Invitrogen (Carlsbad, CA) (catalog no. 12935-100).

Database Sequence Analysis

Putative Sec16 homologues from various species were identified using the Ensembl genome browser (http://www.ensembl.org), and "best guess" predictions of the complete sequences were made using homology considerations plus additional data from the National Center for Biotechnology Information databases (http://www.ncbi.nlm.nih.gov/), Swiss-Prot (http://www.ebi.ac.uk/swissprot/), and the UCSC genome browser (http://genome.ucsc.edu/). Sec16L has previously been designated KIAA0310 in humans or AU024582 in mouse, and Sec16S has been designated RGPR-p117. Homologues of both Sec16L and Sec16S were identified in the databases for human (*Homo sapiens*), mouse (*Mus musculus*), and chicken (*Gallus gallus*) species. Only a single Sec16 homologue was identified for pufferfish (*Tetraodon nigroviridis*), zebrafish (*Danio rerio*), frog (*Xenopus tropicalis*), fruit fly (*Drosophila melanogaster*), fission yeast (*Schizosaccharomyces pombe*), and budding yeast (*S. cerevisiae*). For mustard plant (*Arabidopsis thaliana*), two closely related Sec16 homologues were identified: the predicted 1361-residue protein diagrammed in Figure 1 and a predicted 1350-residue protein.

Sequence alignments were generated with MegAlign software from DNASTAR (Madison, WI) by using the ClustalW algorithm (Thompson *et al.*, 1994).

Cloning of Human Liver Sec16L and Sec16S

cDNA from 5 μg of normal human liver total RNA was prepared using Superscript III reverse transcriptase (Invitrogen). Specific primers were used to amplify a 6465-base pair fragment corresponding to the Sec16L open reading frame and a 3183-base pair fragment corresponding to the Sec16S open reading frame. These polymerase chain reaction (PCR) products were sequenced directly to determine the correct cDNA sequences. The amplified fragments were then cloned downstream of the green fluorescent protein (GFP) gene in pmGFP-C1, which is derived from the expression vector pEGFP-C1 (Clontech, Mountain View, CA) and encodes enhanced GFP with a monomerizing A206K mutation (Zacharias *et al.*, 2002). The cloned genes were sequenced again to confirm that they matched the consensus sequences obtained from the PCR products. For FLAG epitope tagging, the Sec16L and Sec16S genes were subcloned as described below from the pEGFP-C1 vector into pCMV-3Tag-1A (Stratagene, La Jolla, CA), thereby replacing the GFP tag with a triple-FLAG tag. The Sec16L gene was excised as a BglII–EcoRI fragment and subcloned into pCMV-3Tag-1A that had been digested with BamHI and EcoRI, and the Sec16S gene was excised as a BglII–HindIII fragment and subcloned into pCMV-3Tag-1A that had been digested with BamHI and EcoRI.

The cDNA sequences of human liver Sec16L and Sec16S have been submitted to GenBank (accession nos. DQ903855 and EF125213, respectively).

Cell Culture and RNAi Treatment

HeLa cells were grown under standard culture conditions in DMEM with 10% fetal calf serum. For RNAi treatment, cells growing in a 150-mm dish at <50% confluence were trypsinized and seeded into a six-well chamber at a density that yielded <50% confluence after overnight growth. Each well contained 2 ml of medium and a 12-mm coverslip with a well in a silicon gasket (catalog. no. MSR12-0.5; Grace Bio-Labs, Bend, OR). The medium was replaced in the morning with fresh medium lacking antibiotic. After 3 h, the cells were transfected with the appropriate RNAi by using Oligofectamine (Invitrogen) according to the manufacturer's instructions. At 4 h after transfection, the cells were supplemented with serum-containing medium, and at 36 h after transfection, the cells were washed and subjected to either immunofluorescence microscopy or real-time reverse transcription (RT)-PCR.

Fluorescence Microscopy

For immunofluorescence microscopy, a polyclonal antibody against an N-terminal peptide of Sec23A, obtained from Affinity Bioreagents (Golden, CO) (catalog no. PA1-069), was used at 5 μg/ml. (This batch of antibody worked well, but a subsequent batch designated PA1-069A gave a strong nuclear labeling and was therefore unsuitable for immunofluorescence.) Anti-giantin monoclonal antibody (mAb), a generous gift of Dr. Adam Linstedt, was used at 1 μg/ml. Monoclonal anti-GFP antibody from Roche Diagnostics (Indianapolis, IN) (catalog no. 11814460001) was used at 10 μg/ml.

Fluorescence and differential interference contrast imaging of live or fixed cells expressing fluorescent proteins, and image capture and processing were all conducted as described previously (Rossanese *et al.*, 1999; Hammond and Glick, 2000). *z*-stack image volumes of fixed cells were average projected. Figure assembly and adjustment of brightness and contrast were performed using Photoshop (Adobe Systems, Mountain View, CA).

Real-Time RT-PCR

Total RNA was collected from HeLA cells at 36 h after transfection by using the RNeasy kit (QIAGEN, Valencia, CA). Reverse transcription from equal amounts of total RNA was performed using SuperScript III to obtain cDNA. PCR was then performed with oligonucleotides specific for Sec16L (forward primer, CCCGTAGGAGGTGAAACAGA, and reverse primer, CGATCTGCCTCAAATGGTTT), Sec16S (forward primer, TCAGCCTGTGTCTGGAGTTG, and reverse primer, TTGCCTTGGCACTCTTTCTT), Sec12 (forward primer, AGTCCTGTGGCCATGAAGTC, and reverse primer, TCCACAGCCACAGAGAACAG), or actin (forward primer, GGACTTCGAGCAAGAGATGG, and reverse primer, AGCACTGTGTTGGCGTACAG). This procedure used SYBR Green PCR reagents from Applied Biosystems (Foster City, CA) in an ABI7300 real-time PCR machine.

Glutathione S-Transferase (GST) Pull Down and Immunoblot Analysis

HeLa cells were lysed using CytoBuster (Novagen, Madison, WI) in the presence of the Complete Mini EDTA-free protease inhibitor cocktail (Roche Diagnostics). *Escherichia coli* cells expressing GST fusion proteins from the inducible vector pGEX-4T-1 (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) were lysed with BugBuster (Novagen), and the lysate was incubated with glutathione-agarose beads. The beads were washed with a 1:1 mixture of phosphate-buffered saline and ProFound lysis buffer (Pierce Chemical, Rockford, IL), and they were then incubated with the HeLa cell lysate. After additional washes, the GST fusions with their bound HeLa cell proteins were eluted with 100 mM glutathione. The eluted samples were subjected to SDS-PAGE on an 8–16% gradient gel (Pierce Chemical) with PageRuler molecular weight markers (MBI Fermentas, Hanover, MD). Separated proteins were transferred to a polyvinylidine fluoride membrane. For immunodetection, the anti-Sec23A antibody was used at 1 μg/ml. Bands were visualized with a SuperSignal West Femto kit (Pierce Chemical).

Immunoprecipitation

For the experiment depicted in Figure 8, 160-mm dishes of ~90% confluent HeLa cells were transfected with the indicated plasmid pairs by using Lipofectamine 2000 (Invitrogen). At 18 h after transfection, the cells were lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 containing the Complete Mini protease inhibitor cocktail (Roche Diagnostics). The cell lysates were centrifuged for 10 min at 12,000 $\times$ *g* at 4°C. Protein concentrations of the lysates were then measured using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). For each sample, 10% of the lysate was set aside for subsequent SDS-PAGE and immunoblotting. Seven hundred micrograms of each lysate were immunoprecipitated overnight with anti-FLAG affinity gel (catalog no. A2220; Sigma-Aldrich, St. Louis, MO). Bound proteins were eluted with 0.1 M glycine, pH 3.5, and the immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting with anti-GFP polyclonal antibody (catalog no. ab6556; Abcam, Cambridge, MA) or peroxidase-conjugated anti-FLAG mAb (catalog no. A8592; Sigma-Aldrich).

RESULTS

Identification of Mammalian Sec16 Homologues

In budding yeasts, Sec16 contains a central conserved domain of ~420 amino acids and a C-terminal conserved domain of ~165 amino acids (Connerly *et al.*, 2005). Sequence-based homology searches with the central conserved domain revealed putative Sec16 homologues in a variety of eukaryotes, including mammals (Figure 1 and Supplemental Figure 1). Although the extent of conservation is relatively

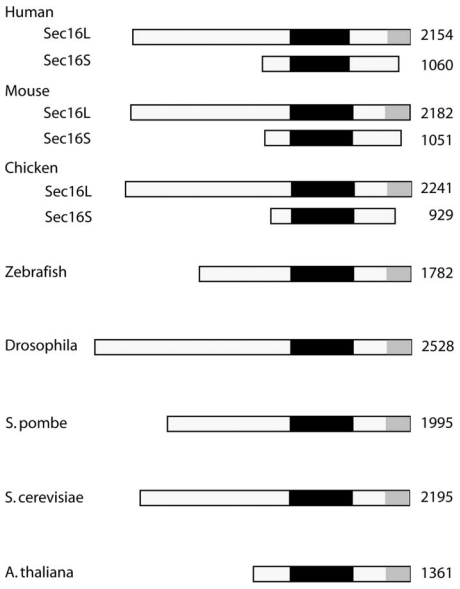

Figure 1. Schematic representations of Sec16 homologues from different species. Diagrammed are putative homologues of Sec16 from human (*H. sapiens*), mouse (*M. musculus*), chicken (*G. gallus*), zebrafish (*D. rerio*), fruit fly (*D. melanogaster*), fission yeast (*S. pombe*), budding yeast (*S. cerevisiae*), and mustard plant (*A. thaliana*). The black box represents the central conserved domain, and the gray box represents the C-terminal conserved domain. Listed on the right are the sizes of the predicted protein products in amino acids.

low, most of the putative Sec16 homologues are characterized by large size, location of the central conserved domain near the middle of the polypeptide chain, and the presence of a C-terminal domain with distant similarity to the C-terminal conserved domain of yeast Sec16 (see below). Interestingly, each of the mammalian genomes that we examined encoded two putative Sec16 homologues: a large protein of ~2000 amino acids that contains both of the conserved domains, and a smaller protein of ~1000 amino acids that seems to contain only the central conserved domain. We tentatively designated these proteins Sec16L and Sec16S, respectively.

Human Sec16L is encoded by the gene KIAA0310 on chromosome 9 (Nagase *et al*., 1997; Nakajima *et al*., 2002). The sequence of our cloned cDNA from human liver predicts a 231-kDa protein of 2154 amino acids. By contrast, the original KIAA0310 cDNA, which was generated from brain, encoded a protein of 2179 amino acids. This difference may reflect tissue-specific alternative splicing (Supplemental Figure 2).

Human Sec16S is encoded by a gene on chromosome 1 and was previously described as a regucalcin gene promoter region-related protein of 117 kDa (RGPR-p117) by Yamaguchi and colleagues, who identified it as a putative DNA-binding protein by using a yeast one-hybrid screen (Misawa and Yamaguchi, 2001). This group reported that RGPR-p117 is present in a variety of vertebrate species and is expressed in multiple tissues (Misawa and Yamaguchi, 2001; Sawada and Yamaguchi, 2005a). Our cloned cDNA from human liver predicts a 1060-amino acid protein that is virtually identical to RGPR-p117, except for a few amino acid substitutions that probably reflect allelic variation.

If Sec16L and Sec16S are true Sec16 homologues, they would be expected to colocalize with COPII components at tER sites. A typical cultured mammalian cell contains several hundred punctate tER sites, some of which are clustered in a juxtanuclear region near the Golgi, and others of which are distributed throughout the peripheral ER network (Bannykh and Balch, 1997; Tang *et al*., 1997; Hammond and Glick, 2000). These tER sites tend to be disrupted by conventional immunofluorescence procedures, but with an improved protocol they can be readily visualized using anti-COPII antibodies (Hammond and Glick, 2000). We transfected HeLa cells with a plasmid for transient expression of GFP-tagged Sec16L or Sec16S and then labeled tER sites in these cells by using an antibody against the COPII coat protein Sec23A (Paccaud *et al*., 1996). Both of the GFP-tagged proteins showed virtually perfect colocalization with Sec23A at 12 h after transfection (Figure 2A). Similar results were obtained using a FLAG epitope tag instead of GFP (Supplemental Figure 3). By 36 h after transfection, the tER sites were smaller and about twice as numerous in cells expressing GFP–Sec16L compared with cells expressing GFP–Sec16S (Supplemental Figure 4), but GFP-Sec16L continued to colocalize with Sec23A (data not shown). The implication of these results is that Sec16L and Sec16S should colocalize. Indeed, YFP-tagged Sec16L colocalized completely with CFP-tagged Sec16S at various times after transfection (Figure 2B; data not shown). These findings are reminiscent of the colocalization of Sec16 with COPII coat proteins in budding yeasts (Connerly *et al*., 2005), and they support the interpretation that Sec16L and Sec16S are functional homologues of yeast Sec16.

An obvious question was whether Sec16L and Sec16S are alternative isoforms with tissue-specific expression. We used real-time RT-PCR to quantify the expression of the two genes in a variety of normal human tissues (Figure 2C). The mRNA levels of Sec16L were comparable in all of the tissues examined, although somewhat higher levels were observed in pancreas. In Sec16S, mRNA levels were nearly the same in all of the tissues examined. The levels of the protein products remain to be determined. Nevertheless, these data suggest that Sec16L and Sec16S are both expressed in multiple tissues and might therefore have nonredundant functions.

Depleting Sec16L or Sec16S Disrupts tER Sites and Blocks ER Export

In *P. pastoris*, a point mutation in the central conserved domain of Sec16 causes fragmentation of tER sites (Connerly *et al*., 2005). This effect is not due simply to inhibition of ER export, because blocking ER export with a dominant-negative version of Sar1 fails to disrupt tER sites (Connerly *et al*., 2005). To test whether the mammalian Sec16 homologues are required for normal tER organization, we depleted HeLa cells of either Sec16L or Sec16S by using RNAi-mediated knockdown of gene expression. The RNAi molecules were designed to match coding regions that show no similarity in the two homologues. As a negative control, we blocked ER export (see below) by depleting the single human homologue of Sec12 (Weissman *et al*., 2001). Delocalizing *P. pastoris* Sec12 from tER sites has little effect on tER organization (Soderholm *et al*., 2004), and mammalian Sec12 is not concentrated at tER sites (Weissman *et al*., 2001), so we expected that Sec12 would be dispensable for tER organization in HeLa cells.

RNAi directed against Sec16L dramatically reduced the levels of Sec16L mRNA, with no detectable effect on the mRNA levels for Sec16S or Sec12 (Figure 3A). Similar specific reductions were seen with RNAi against Sec16S or Sec12 (Figure 3A). The analyses of RNAi-treated cells were done at 36 h after transfection because at this time point, there was a strong effect on mRNA levels, yet most of the

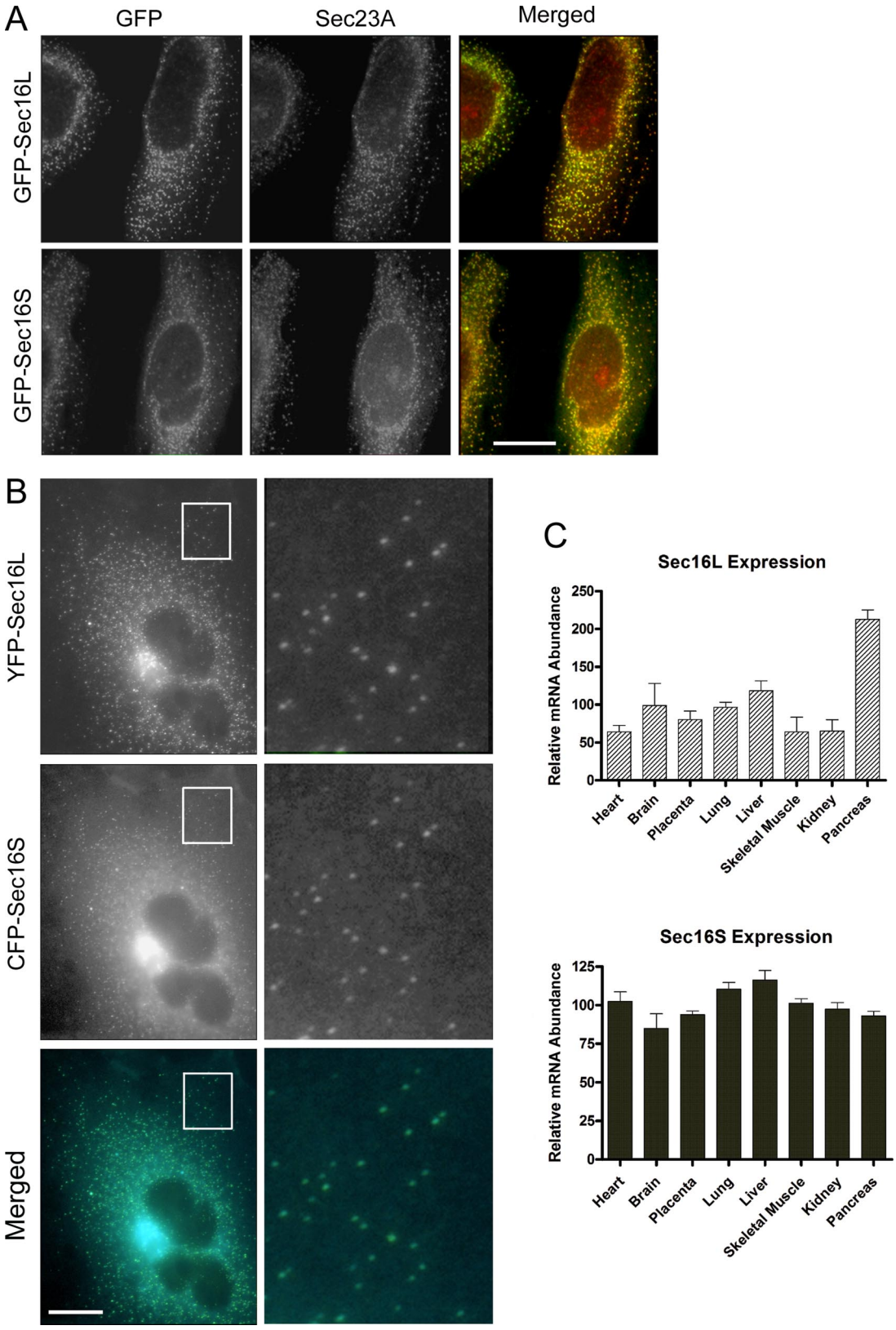

Figure 2. Sec16L and Sec16S colocalize with a COPII coat protein and with each other and are both expressed in a variety of tissues. (A) Colocalization of the Sec16 homologues with Sec23A. A plasmid encoding GFP-Sec16L (top) or GFP-Sec16S (bottom) was transiently transfected into HeLa cells. At 12 h after transfection, the cells were processed for immunofluorescence by using anti-GFP mAb plus anti-Sec23A polyclonal antibody. The merged images show colocalization of GFP (green) with Sec23A (red). Bar, 20 μm. (B) Colocalization of the Sec16 homologues with each other. A plasmid encoding YFP-Sec16L was cotransfected into HeLa cells with a plasmid encoding CFP-Sec16S. At 12 h after transfection, the cells were fixed and subjected to fluorescence microscopy. The panels on the right show an

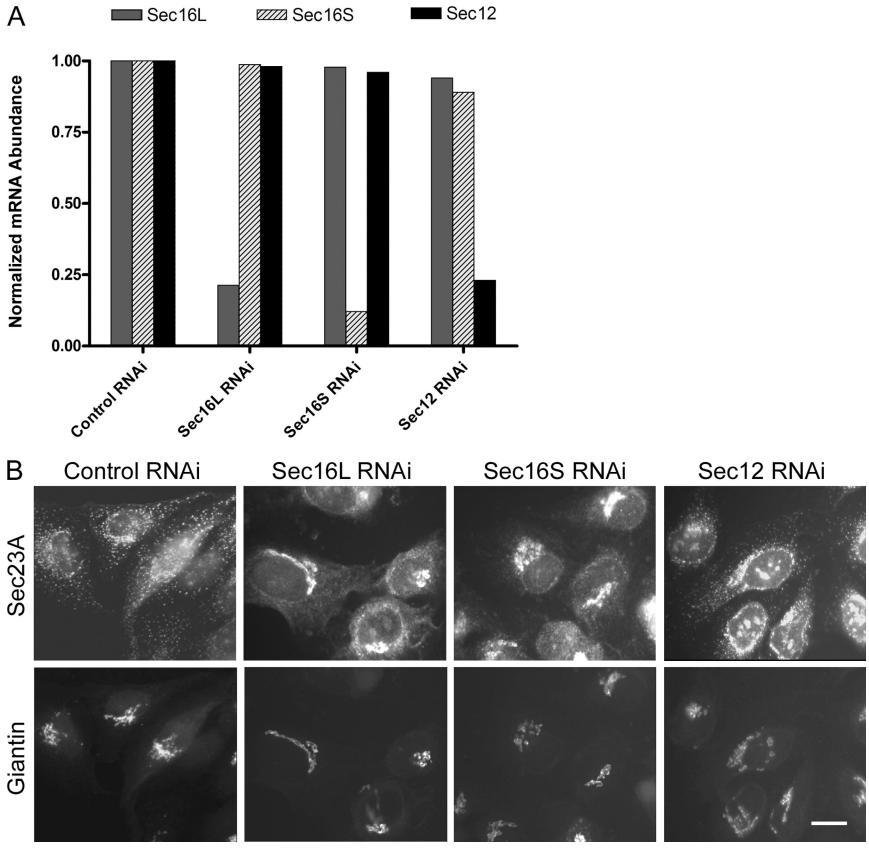

Figure 3. RNAi-mediated knockdown of Sec16L or Sec16S disrupts tER sites. (A) Quantitation of RNAi-mediated knockdowns. HeLa cells cultured on coverslips were transfected either with a control nonspecific RNAi or with an RNAi against Sec16L, Sec16S, or Sec12. At 36 h after transfection, total RNA from each sample was subjected to real-time RT-PCR by using primers specific for Sec16L, Sec16S, or Sec12. The linear phase yields were normalized against actin mRNA signals, and the signals obtained with the control RNAi were defined as 1.0. (B) tER and Golgi patterns in RNAi-treated HeLa cells. The same cells used in A were assayed by immunofluorescence with a polyclonal antibody against the COPII protein Sec23A (top) plus a mAb against the Golgi protein giantin (bottom). Indicated at the top are the RNAi duplexes used for the transfections. Bar, 20 μm.

cells retained their normal appearance. At 48 h after transfection, the cells treated with a nonspecific control RNAi retained their normal appearance, but many of the cells treated with the specific RNAi molecules were detaching from the coverslip (data not shown), indicating that the RNAi-mediated knockdowns were eventually toxic. This observation, together with the results described below, indicates that the reductions in mRNA levels of Sec16L, Sec16S, and Sec12 caused functionally significant depletions of the corresponding proteins.

After transfection with either Sec16L or Sec16S RNAi, the punctate tER pattern was abolished in essentially all of the cells (Figure 3B). Although Sec23A was still detectable in the juxtanuclear Golgi region, the punctate pattern was replaced with more homogeneous staining. In the same cells, we visualized the Golgi with an antibody against the membrane-anchored coiled-coil protein giantin (Linstedt and Hauri, 1993). Treatment with RNAi against Sec16L or Sec16S did not substantially alter the juxtanuclear Golgi staining (Figure 3B). Similar results were obtained with a second pair of RNAi duplexes directed against Sec16L or Sec16S (data not shown). Moreover, simultaneous treatment with RNAi against Sec16L and Sec16S gave results indistinguishable from treatment with RNAi against either protein alone (Supplemental Figure 5). Treatment with RNAi against Sec12 resulted in tER sites that labeled less intensely than in the control sample, but did not alter the number or distribution of tER sites (Figure 3B). Therefore, the tER disruption that results from depleting Sec16L or Sec16S is probably a specific effect rather than a consequence of blocking COPII vesicle formation. These results indicate that both Sec16L and Sec16S are required for normal tER organization in HeLa cells.

In *S. cerevisiae*, Sec16 is required for COPII-mediated ER export (Espenshade *et al.*, 1995). To test whether the mammalian Sec16 proteins have a similar function, we examined ER export in cells that had been treated with RNAi against Sec16L or Sec16S. Sec12 served once again as a control, but this time it was a positive control because Sec12 depletion should block COPII vesicle formation. To assay for ER export, we used a HeLa cell line that stably expresses a GFP fusion to the Golgi protein *N*-acetylgalactosaminyltransferase-2 (GalNAc-T2-GFP; Storrie *et al.*, 1998). It was previously shown that when this cell line is incubated in the presence of brefeldin A (BFA), GalNAc-T2-GFP redistributes to the ER, and when BFA is subsequently removed, GalNAc-T2-GFP is exported from the ER and returns to the reformed Golgi (Kapetanovich *et al.*, 2005). We confirmed those observations in cells that were treated with a nonspecific RNAi. At 30 min after BFA addition, GalNAc-T2-GFP had redistributed to the ER (Figure 4A). At 1 h after BFA removal, ~50% of the cells showed concentration of GalNAc-T2-GFP in post-ER compartments, and by 3 h after BFA removal, >80% of the cells had restored a normal Golgi pattern of

Figure 2 (cont). enlarged view of the inset in the panels on the left. The merged images show colocalization of YFP-Sec16L (green) with CFP-Sec16S (blue). Bar, 10 μm. (C) Tissue mRNA levels of the Sec16 homologues. Total RNA samples from the indicated human tissues were subjected to real-time RT-PCR by using primers specific for Sec16L or Sec16S. The linear phase yields were normalized against actin mRNA signals. The resulting numbers were then normalized again by defining the average signal from all of the tissues as 100. Bars indicate SEs of the mean calculated from three separate experiments.

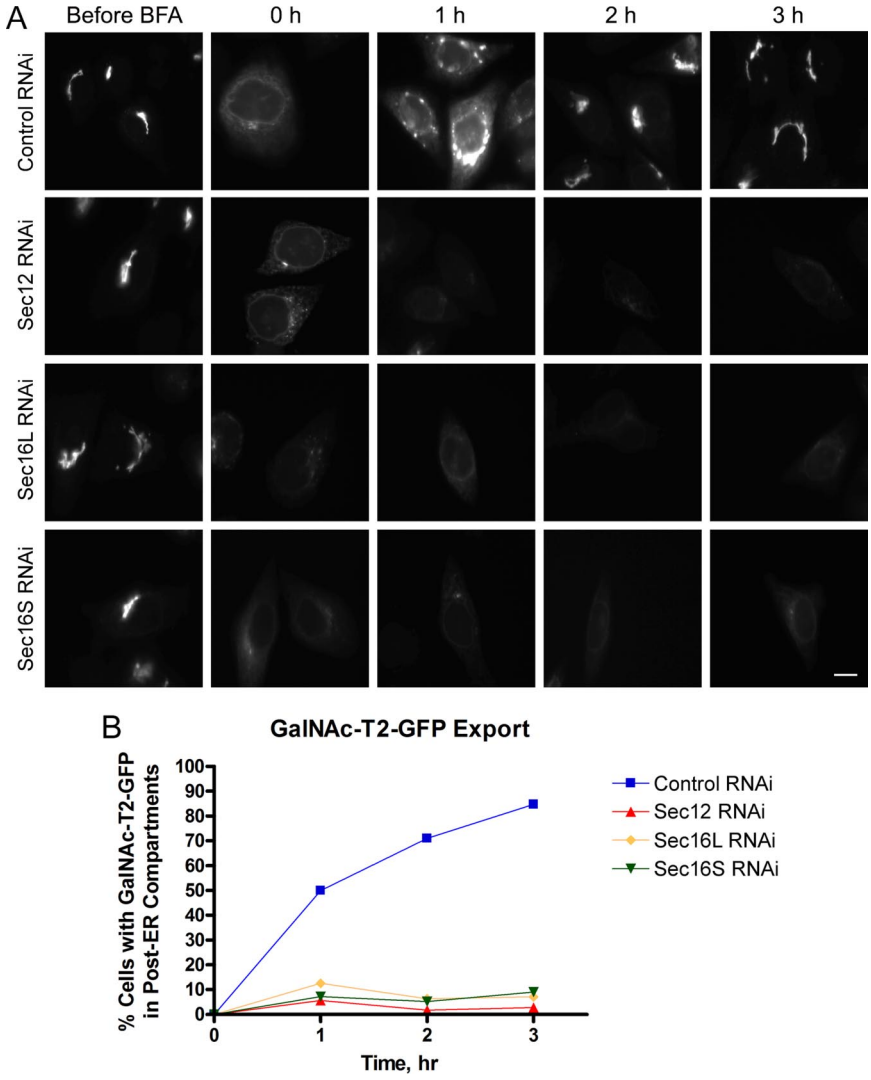

Figure 4. RNAi-mediated knockdown of Sec16L or Sec16S disrupts ER export. (A) ER export in RNAi-treated cells. HeLa cells stably expressing GalNAc-T2-GFP were transfected either with a control nonspecific RNAi or with an RNAi against Sec16L, Sec16S, or Sec12. At 36 h after transfection, the cells were treated with 5 μg/ml BFA for 30 min to redistribute GalNAc-T2-GFP into the ER. The cells were then washed in BFA-free medium, and at the indicated times after BFA removal, samples were fixed for fluorescence microscopy to assay for export of GalNAc-T2-GFP to post-ER compartments. All of the images in this figure were taken at the same exposure and processed in parallel. Bar, 20 μm. (B) Quantitation of the experiment shown in A. At each time point after transfection with the indicated RNAi, 100 cells were scored for the presence or absence of GalNAc-T2-GFP in post-ER compartments.

GalNAC-T2-GFP (Figure 4, A and B). By contrast, RNAi-mediated depletion of Sec16L, Sec16S, or Sec12 almost completely blocked the ER export of GalNAc-T2-GFP and its accumulation in the juxtanuclear Golgi region (Figure 4, A and B). Therefore, both Sec16L and Sec16S are required for ER export in HeLa cells.

tER Localization Depends on N-Terminal Regions of Sec16L and Sec16S

It seems likely that the tER localization of Sec16L and Sec16S involves evolutionarily conserved interactions with COPII components. We therefore predicted that the central conserved domains of the Sec16 homologues would be important for localization. Surprisingly, the primary localization determinant in each protein was not the central conserved domain (Supplemental Figure 6), but instead was located upstream of this domain.

In Sec16L, a series of deletion constructs indicated that a region of ~300 amino acids upstream of the central conserved domain was essential for tER localization (Figure 5A). A GFP fusion to this region of Sec16L localized to tER sites, and deletion of this region from full-length Sec16L abolished tER localization (Figure 5C). These results were very consistent from cell to cell. Similarly, a 200-amino acid region of Sec16S upstream of the central conserved domain was sufficient and necessary for tER localization (Figure 5, B and C). These tER localization regions show no detectable homology between Sec16L and Sec16S, raising the possibility that the two proteins localize by different mechanisms. Although we do not yet know the molecular functions of the tER localization regions, our data indicate that Sec16 proteins have important interactions outside of the previously identified conserved domains.

A C-Terminal Sec23-binding Domain Is Present in Sec16L but Not in Sec16S

BLAST searches revealed that Sec16 homologues from plants to fungi to mammals show some sequence conservation near the C terminus, particularly in a stretch of ~50–60 amino acids at the beginning of the C-terminal conserved domain (Figure 6A). Interestingly, this conservation is detectable in Sec16L but not in Sec16S. During our analysis of tER localization regions, we noticed that transient expression of the C-terminal conserved domain of Sec16L strongly disrupted tER sites as marked by Sec23A and also disrupted the Golgi as marked by giantin (Figure 6B). By contrast, expression of a C-terminal fragment of Sec16S had no such

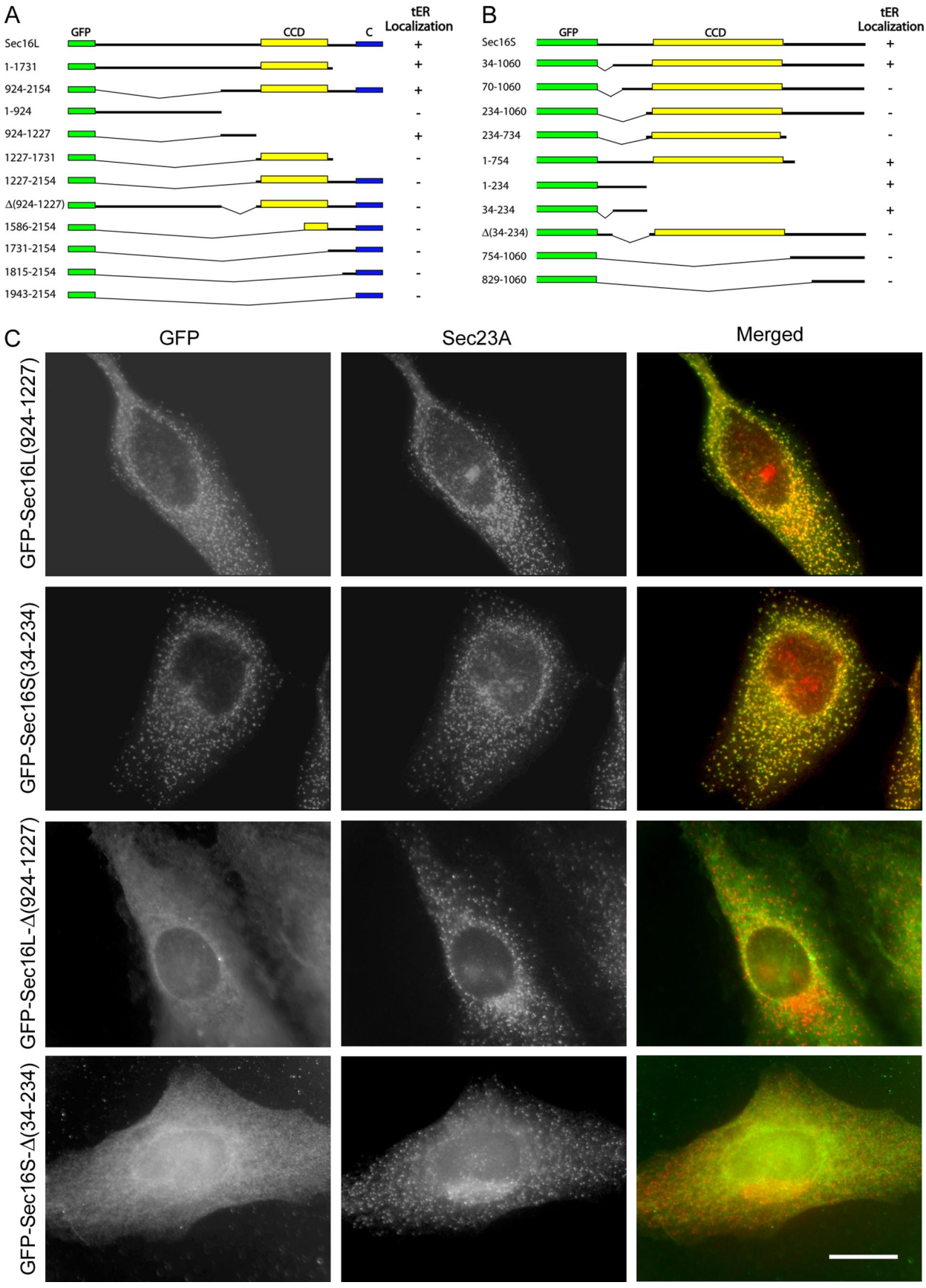

Figure 5. Mapping of tER localization determinants in Sec16L and Sec16S. (A) Deletion analysis of Sec16L. The starting construct was full-length Sec16L tagged at its N terminus with GFP (green box). The central conserved domain (CCD, residues 1267-1713) is shown as a

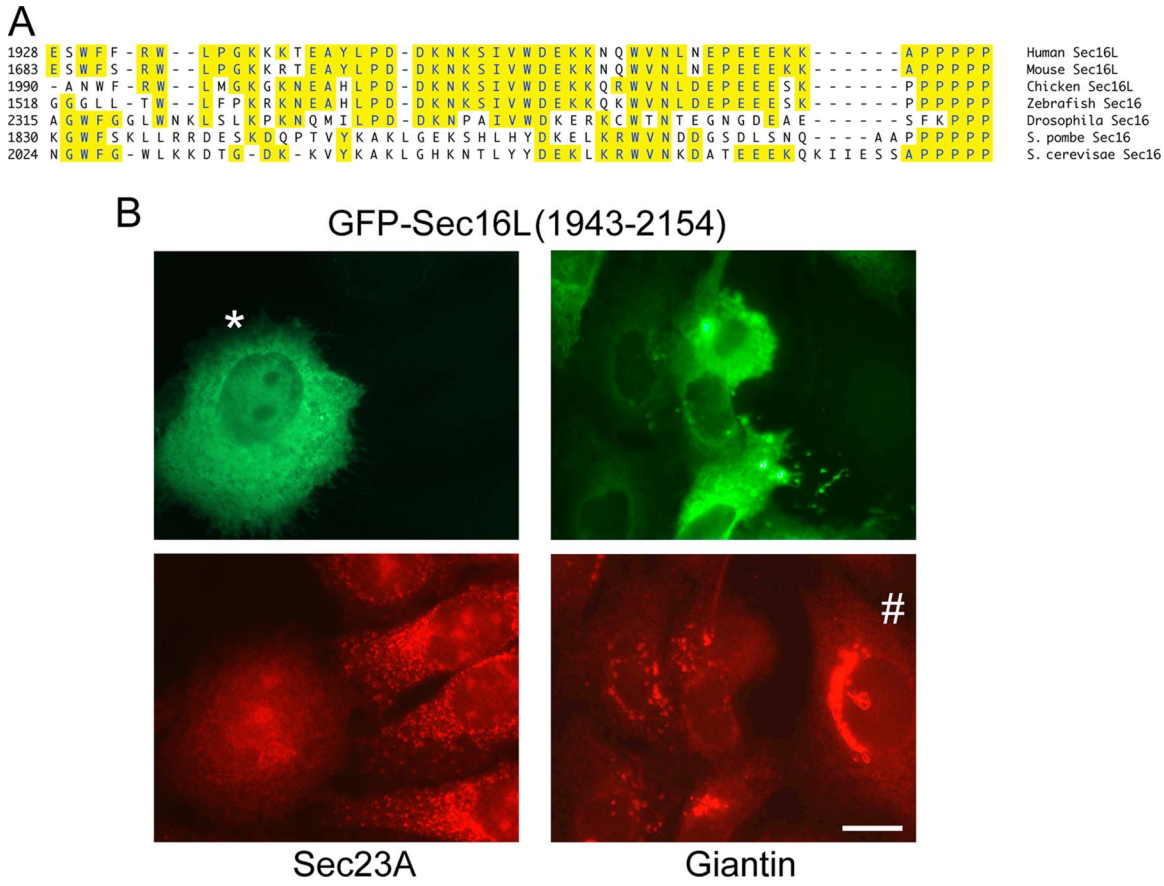

Figure 6. Expression of the C-terminal conserved domain of Sec16L disrupts tER and Golgi compartments. (A) Sequence alignment showing a portion of the C-terminal conserved domain of Sec16L or Sec16 from each of the indicated species. Residues matching the consensus are highlighted in yellow. (B) Expression of a fusion between GFP and the C-terminal conserved domain of Sec16L (residues 1943-2154) in HeLa cells. At 36 h after transfection, cells were subjected to immunofluorescence microscopy to visualize GFP (green) plus either Sec23A or giantin (red). In the field on the left, the single transfected cell is marked with an asterisk (*) and is the only visible cell with a disrupted Sec23A pattern. In the field on the right, the single nontransfected cell is marked with a cross-hatch (#) and is the only visible cell with a normal giantin pattern. Bar, 20 μm.

effect (data not shown). These observations suggest that the C-terminal conserved domain of Sec16L interacts with a partner protein, probably a COPII component.

A likely candidate for such a partner protein is Sec23A, because in *S. cerevisiae* Sec16, a region overlapping the C-terminal conserved domain binds to Sec23 (Espenshade *et al.*, 1995). To test for an interaction with Sec23A, we fused GST to the C-terminal conserved domain of Sec16L. The resulting fusion protein, designated GST-Sec16L(1943-2154), was immobilized on glutathione-agarose beads, and then a HeLa cell lysate was incubated with the beads. Control incubations used either GST alone or the GST-Sec16S(754-1060) fusion protein, which contained the portion of Sec16S downstream of the central conserved domain. As judged by immunoblotting (Figure 7A), Sec23A bound to GST-Sec16L(1943-2154) but not to GST alone or GST-Sec16S(754-1060). The binding to Sec23A was probably direct because it could be detected in a yeast two-hybrid assay. In this experiment (Figure 7B), the cells in rows 1 and 4–6 contained negative control plasmid combinations, and the cells in rows 2 and 3 expressed a Gal4–Sec23A fusion plus either a Gal4-Sec16L(1943-2154) fusion or a Gal4-Sec16S(754-1060) fusion. Two-hybrid interactions were indicated by growth on plates lacking either histidine or adenine. Sec23A showed a strong interaction with Sec16L(1943-2154) but no interaction with Sec16S(754-1060).

These various negative results with the C-terminal portion of Sec16S must be interpreted with caution because we have not demonstrated that this protein fragment is folded or functional. Nevertheless, our combined data support the

Figure 5 (cont). yellow box, and the C-terminal conserved domain (C, residues 1928-2154) is shown as a blue box. Deletions are represented by thin angled lines. The numbers at the left indicate the residues that remained after the deletion, except that Δ(924-1227) indicates the residues that were deleted. tER localization was analyzed by transient transfection into HeLa cells followed by immunofluorescence visualization of GFP and Sec23A (see Figure 2A). A + indicates strong colocalization of the construct with Sec23A, and a − indicates little or no colocalization. (B) Deletion analysis of Sec16S. The methodology was as in part (A). Residues 271-713 make up the central conserved domain. (C) tER localization conferred by small regions of Sec16L and Sec16S. In the top two rows, GFP was fused to residues 924-1227 of Sec16Lor to residues 34-224 of Sec16S. In the bottom two rows, GFP was fused to full-length Sec16L lacking residues 924-1227 or to full-length Sec16S lacking residues 34-224. Immunofluorescence of transiently transfected cells was performed as in Figure 2A. The merged images show localization of the GFP fusion proteins (green) relative to Sec23A (red). Bar, 20 μm.

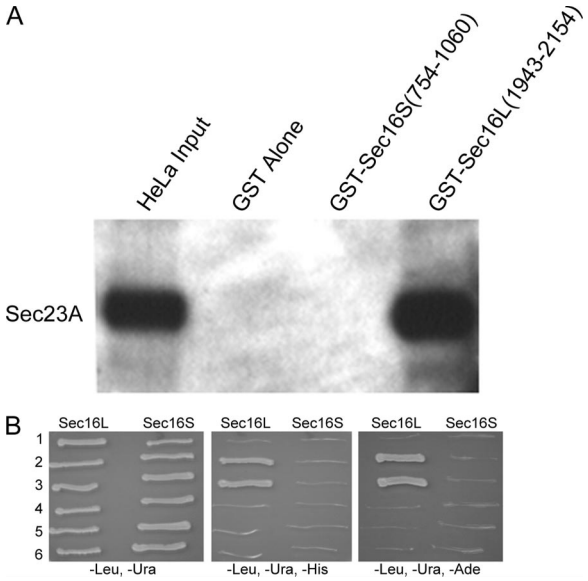

Figure 7. The C-terminal conserved domain of Sec16L interacts with Sec23A. (A) GST pull-down analysis. A GST fusion to a C-terminal fragment of Sec16L (residues 1943-2154) or Sec16S (residues 754-1060) was expressed in bacteria, and the cells were lysed and incubated with glutathione-agarose beads to bind the fusion protein. The beads were then incubated with a HeLa cell lysate. Bound proteins were eluted with glutathione and separated by SDS-PAGE, followed by immunoblotting with anti-Sec23A antibody. The input lane represents 40% of the amount of HeLa cell lysate that was used. (B) Yeast two-hybrid analysis. Gal4 fusions were expressed using a *URA3*"bait" vector and a *LEU2*"prey" vector (James *et al*., 1996). The C-terminal fragments used for the fusions were residues 1943-2154 of Sec16L and residues 754-1060 of Sec16S. The left panel shows an agar plate that selected for the presence of both bait and prey plasmids, whereas the other two panels show plates that also selected for two-hybrid interactions. Row 1, empty bait vector, prey vector expressing a fusion to Sec23A. Row 2, bait vector expressing a fusion to a C-terminal fragment of Sec16L or Sec16S, prey vector expressing a fusion to Sec23A. Row 3, bait vector expressing a fusion to Sec23A, prey vector expressing a fusion to a C-terminal fragment of Sec16L or Sec16S. Row 4, bait vector expressing a fusion to Sec23A, empty prey vector. Row 5, bait vector expressing a fusion to a C-terminal fragment of Sec16L or Sec16S, empty prey vector. Row 6, empty bait vector, prey vector expressing a fusion to a C-terminal fragment of Sec16L or Sec16S.

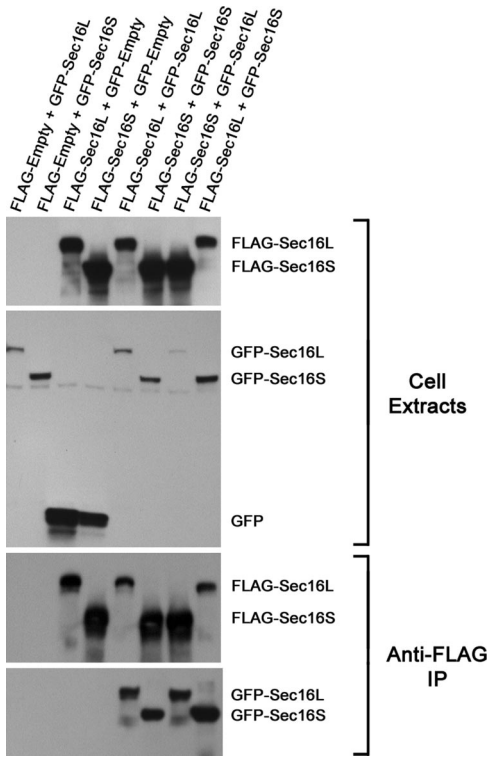

Figure 8. Sec16L and Sec16S are apparently present in multiple copies in a heteromeric complex. HeLa cells were transfected with a plasmid encoding either GFP alone (GFP-Empty), GFP-Sec16L, or GFP-Sec16S, and were simultaneously transfected with a second plasmid encoding either a FLAG tag alone (FLAG-Empty), FLAG-Sec16L, or FLAG-Sec16S. At 18 h after transfection, cell extracts were prepared. In the top two panels, 60 μg of soluble cell extract protein was run in each lane of an SDS-PAGE gel, and the samples were then subjected to immunoblotting with either anti-FLAG antibody (top) or anti-GFP antibody (second panel from top) to confirm that the expected proteins had been produced. In the bottom two panels, the extracts were subjected to immunoprecipitation with anti-FLAG antibody, followed by immunoblotting with either anti-FLAG antibody (second panel from bottom) or anti-GFP antibody (bottom panel). Additional lanes from the bottom two gels (data not shown) revealed that the efficiency of immunoprecipitation of the FLAG-tagged proteins was 15–20%, and the efficiency of coimmunoprecipitation of the GFP-tagged proteins was also 15–20%.

interpretation that Sec16L contains a C-terminal Sec23-interacting domain, whereas Sec16S lacks such a domain.

Sec16L and Sec16S Are Present Together in a Multiprotein Complex

Because the Sec16L and Sec16S genes are both expressed in all tissues examined, and because similar results were obtained by knocking down either Sec16L or Sec16S, we speculated that Sec16L and Sec16S might function together in a heteromeric complex. As an initial test of this hypothesis, we coexpressed FLAG-tagged Sec16L with either GFP–Sec16L or GFP–Sec16S. Both of the GFP-tagged Sec16 proteins were efficiently coimmunoprecipitated with FLAG–Sec16L (Figure 8). Similarly, both of the GFP-tagged Sec16 proteins could be efficiently coimmunoprecipitated with FLAG-tagged Sec16S (Figure 8). These results suggest that a stable heteromeric complex contains multiple copies each of Sec16L and Sec16S. Further investigation will be needed to determine the size and composition of this complex.

DISCUSSION

Previous molecular studies of the secretory pathway have revealed a high degree of conservation between yeast and mammals (Duden and Schekman, 1997). At the stage of ER export, mammalian homologues have been described for Sec12, Sar1, Sec23, Sec24, Sec13, and Sec31 (Kuge *et al*., 1994; Paccaud *et al*., 1996; Tang *et al*., 1997, 1999; Weissman *et al*., 2001; Stankewich *et al*., 2006), and all of these proteins seem to function similarly to their yeast counterparts. However, mammalian cells often have more isoforms of a given COPII component. For example, *S. cerevisiae* contains a single gene each for Sar1, Sec23, and Sec31, but human cells have two genes for each of these proteins. The diversity of mammalian COPII components may be further enhanced by alternative splicing (Stankewich *et al*., 2006).

The present work indicates that the similarity between yeast and mammalian COPII systems extends to Sec16. Five lines of evidence indicate that the proteins we have designated Sec16L and Sec16S are functional homologues of yeast

Sec16. First, the mammalian Sec16 homologues contain internal domains with sequence similarity to the central conserved domain of yeast Sec16 (Supplemental Figure 1). Second, Sec16L contains a C-terminal domain that resembles the C-terminal conserved domain of yeast Sec16 with regard to both its sequence and its ability to bind Sec23 (Figures 6 and 7). Third, like yeast Sec16, the mammalian Sec16 homologues colocalize with COPII coat proteins (Figure 2). Fourth, like *P. pastoris* Sec16, the mammalian Sec16 homologues play a role in tER organization (Figure 3). Fifth, like *S. cerevisiae* Sec16, the mammalian Sec16 homologues are required for ER export (Figure 4).

Sec16S was previously identified using a yeast one-hybrid assay as a binding protein for the regucalcin gene promoter, and it was given the name RGPR-p117 (Misawa and Yamaguchi, 2001). Recombinant RGPR-p117 did not show detectable binding to the regucalcin gene promoter in vitro (Yamaguchi *et al*., 2003), but overexpression of RGPR-p117 in normal rat kidney cells increased regucalcin levels by ~25% (Sawada and Yamaguchi, 2005b), consistent with a role for RGPR-p117 in regulating regucalcin expression. Alternatively, the original one-hybrid result with the regucalcin gene promoter may have been a false positive. Although tagged RGPR-p117 was reportedly localized to the nucleus of normal rat kidney cells as judged by immunofluorescence (Sawada *et al*., 2005), the images include juxtanuclear staining that is consistent with the tER localization observed here. We think that the combined data favor the idea that the protein previously described as RGPR-p117 actually has a primary function in ER export. Therefore, it seems appropriate to rename this protein to Sec16S.

While this manuscript was in revision, an article was published describing a mammalian Sec16 homologue that corresponds to Sec16L (Watson *et al*., 2006). The results from that study are substantially in agreement with ours. We suggest that Sec16L can be viewed as a canonical Sec16 protein because it contains a C-terminal conserved domain, which can also be detected in Sec16 homologues from invertebrates, fungi, and plants (Figure 1). By contrast, Sec16S may be a recent evolutionary invention. The chicken genome contains homologues of both Sec16L and Sec16S, but the genome databases for the frog *X. tropicalis*, the zebrafish *D. rerio*, and the pufferfish *T. rubripes* contain only Sec16L homologues. This observation suggests that Sec16S is specific to amniote vertebrates, and arose after the divergence that separated the mammalian and bird lineages from the amphibian and fish lineages (Meyer and Zardoya, 2003).

We favor the idea that in mammals, both Sec16L and Sec16S are essential components of a heteromeric "Sec16 complex." This hypothesis can explain the following observations: tagged Sec16L and Sec16S colocalized completely (Figure 2); mRNA for both Sec16L and Sec16S was found in all tissues examined (Figure 2); RNAi-mediated knockdown of either Sec16L or Sec16S inhibited ER export (Figure 4); and knockdown of either Sec16L or Sec16S or of both proteins together produced the same type of tER disruption (Figure 3 and Supplemental Figure 5). Moreover, our coimmunoprecipitation data suggest the existence of a stable complex containing at least two copies each of Sec16L and Sec16S (Figure 8). It seems likely that other organisms also have an oligomeric Sec16 complex. Indeed, there is evidence that *S. cerevisiae* Sec16 oligomerizes (Espenshade, personal communication). Given that individual Sec16 polypeptides are typically quite large, the putative Sec16 complex may be a high-molecular-weight particle. These speculative ideas can be tested by extending the biochemical analysis of the Sec16L–Sec16S association.

It is presently unclear why mammalian cells contain two distinct Sec16 homologues. Insight may come from examining the mammalian secretory pathway to identify features that are absent in most other eukaryotes. For example, mammalian cells are unusual in that the Golgi stacks are linked into a juxtanuclear Golgi ribbon that is spatially separated from many of the tER sites (Bannykh and Balch, 1997; Rambourg and Clermont, 1997). However, *Xenopus* cells also have a juxtanuclear Golgi ribbon (Le Bot *et al*., 1998), but they seem to lack Sec16S, suggesting that Sec16S is crucial for some other aspect of the mammalian tER–Golgi system.

Although the functions of Sec16 remain mysterious, this protein interacts with a variety of partners (Gimeno *et al*., 1995, 1996; Shaywitz *et al*., 1997) and seems to be a key player in choreographing the interactions that underlie the dynamics of COPII vesicles and tER sites. *S. cerevisiae* Sec16 is required for COPII vesicle formation (Espenshade *et al*., 1995; Supek *et al*., 2002). In *P. pastoris*, Sec16 seems to be an order of magnitude less abundant at tER sites than COPII coat subunits, suggesting that Sec16 is a regulator of COPII vesicle assembly rather than a stoichiometric subunit of the COPII coat protomer (Connerly *et al*., 2005). *P. pastoris* Sec16 has been implicated in tER site formation, and it may serve to link COPII vesicle formation to the higher order process of clustering COPII components at tER sites (Connerly *et al*., 2005). Here, we offer evidence that the mammalian Sec16 homologues have similar functions.

As an initial step toward understanding these functions, we are analyzing the individual domains of yeast and mammalian Sec16. The C-terminal conserved domain of *S. cerevisiae* Sec16 binds Sec23 and is essential for cell viability (Espenshade *et al*., 1995). Similarly, the C-terminal conserved domain of Sec16L binds Sec23A (Figure 7), and expression of this isolated domain disrupts tER and Golgi organization (Figure 6), consistent with a physiologically significant role for the C-terminal conserved domain. The central conserved domain serves as the primary signature of Sec16 homologues at the sequence level (Figure 1), and it likely also has an important function. Surprisingly, neither of these conserved domains seems to be a major determinant of tER localization. Instead, regions of Sec16L and Sec16S upstream of the central conserved domain are necessary and sufficient for tER localization (Figure 5). *P. pastoris* Sec16 also localizes by means of an N-terminal region upstream of the central conserved domain, and fungal Sec16 homologues show stretches of conservation in this N-terminal region, suggesting the existence of a functionally conserved domain that was not identified in our earlier analysis (Liu, unpublished data). It is therefore intriguing that Sec16L and Sec16S show no detectable similarity in their tER localization regions. Perhaps only Sec16L or Sec16S has a true tER localization signal, and the N-terminal region in the other homologue mediates binding to a partner protein in the Sec16 complex. Further analysis will be needed to determine whether the N-terminal regions of Sec16L and/or Sec16S are functionally related to the tER localization region of *P. pastoris* Sec16.

ACKNOWLEDGMENTS

We thank Peter Espenshade and members of the Glick laboratory for helpful discussion and Adam Linstedt for providing anti-giantin antibody. This work was supported by National Institutes of Health Grant GM-61156.

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